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24 h urinary free cortisol in large-scale epidemiological studies: Short-term and long-term stability and sources of variability

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Summary
Background: Function of the hypothalamus—pituitary—adrenal (HPA) axis has been associated with several somatic and psychiatric health problems. The amount of free cortisol excreted in the urine during 24 h (24-h UFC) has often been used as a proxy for HPA-axis function. Reference values for 24-h UFC and their stability in the short and long term, as well as sources of variability, are largely lacking.
Methods: This study was performed in a general population cohort. Participants collected 24-h UFC on two consecutive days (T1), and repeated this collection approximately 2 years later (T2). Cortisol in urine was measured using LC—MS/MS. Height and weight were measured at the research facilities; glomerular filtration rate was estimated using creatinine clearance. Psychological distress (General Health Questionnaire), smoking, alcohol use and exercise were measured by means of questionnaires.

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Results: 24-h UFC stability on a day-to-day basis was 0.69 (T1, $N = 1192$) and 0.72 (T2, $N = 963$) (both $p < 0.001$). Long-term stability as indicated by correlation between 2-day averages of T1 and T2 was 0.60 ($N = 972$, $p < 0.001$). Multivariable linear regression analysis revealed that 24-h UFC was predicted by urine volume (standardized beta 0.282 (T1, $N = 1556$) and 0.276 (T2, $N = 1244$); both $p < 0.001$) and glomerular filtration rate (standardized beta 0.137 (T1) and 0.179 (T2); both $p < 0.001$), while also sex explained a small part (standardized beta for female sex −0.057 (T1) and −0.080 (T2); both $p < 0.05$).
Conclusion: 24-h UFC is moderately stable both in the short and the long term. The effects of urine volume and glomerular filtration rate on 24-h UFC are much stronger than those of sex.

1. Introduction

HPA-axis activity is investigated as an etiological factor in a variety of diseases, including the metabolic syndrome, depression, and functional somatic syndromes. The assessment of HPA-axis activity in epidemiological studies is usually done by measuring its end-product cortisol. Under basal conditions, cortisol is predominantly bound to cortisol-binding globulin (also named transcortin), while a small amount is bound to albumin with weak affinity. The fraction of cortisol in the plasma is filtered by the kidney, in which the vast majority is reabsorbed by the proximal tubule. A small fraction of unconjugated cortisol is excreted unchanged in urine.

The amount of (free) cortisol excreted in urine during 24 h (24-h UFC) has often been used as a proxy for HPA-axis function (Heitkemper et al., 1996; Seeman et al., 1997; Griep et al., 1998; Oldehinkel et al., 2001; Cleare et al., 2001; Bierer et al., 2006; Chamarthi et al., 2007; Penninx et al., 2007; Simeon et al., 2007; Tak et al., 2009). Measurement of 24-h UFC excretion has the advantage of being unaffected by short-term fluctuations in cortisol and by varying plasma protein binding capacities (Remer et al., 2008). 24-h UFC is thought to reflect overall daily cortisol production and is, compared to serial blood sampling, relatively easy to collect in a large study population. An important advantage of this method is its reliability (Kushnir et al., 2003).

Several studies found associations between 24-h UFC and somatic or psychiatric pathology (Oldehinkel et al., 2001; Vogelzangs et al., 2010; Tak et al., 2011). When interpreting these findings, one should realize that these types of analyses not only assume that there is variation in 24-h UFC secretion between individuals, but also that individual 24-h UFC secretion is a relatively stable trait over time within individuals. It is questionable whether this latter assumption holds true, since the long- and short-term stability of 24-h UFC have rarely been studied. Moreover, the sources of variation in 24-h UFC are largely unknown.

One potential determinant of the total 24-h UFC is urine volume. Previous studies on the relation between urine volume and the total urinary free cortisol excretion are equivocal, with some studies suggesting a positive relation whereas others found no relation (Fenske, 2006). A potential explanation for these inconsistencies is that urinary free cortisol values that have been determined by some of the assays may represent the sum of cortisol and cortisone (Fenske, 2004). One experimental study found an elevation of urinary free cortisol and urinary free cortisone excretion after water loading, albeit with a more prominent stimulation of urinary free cortisone than free cortisol excretion (Fenske, 2006). This was suggested to be the consequence of augmented formation of cortisone from cortisol, the latter escaping from metabolism or reabsorption in the proximal tubule. Previous associations between urine volume and 24-h UFC might thus be largely explained by cross-reactivity, and it remains unknown whether a true association between urine volume and 24-h UFC exists.

Another source of variation is renal function. Since plasma free cortisol is filtered through the glomeruli with partial tubular reabsorption, the amount of free cortisol appearing in the urine is theoretically dependent on the glomerular filtration rate. Indeed, in a previous study of the relationship between glomerular filtration rate and urinary cortisol excretion it was shown that patients with moderate and especially severe renal impairment had significantly lower 24-h UFC excretion rates than those with no or mild renal impairment (Chan et al., 2004). It is currently unclear whether normal variation in renal function is associated with 24-h UFC.

Sex (Raven and Taylor, 1996), age (VanCauter et al., 1996), smoking (Badrick et al., 2007), alcohol consumption (Thayer et al., 2006), exercise frequency (Luger et al., 1987), stressful events (Schubert et al., 2012) and body mass index (BMI) (Ukkola et al., 2001) may also be responsible for variance in 24-h UFC excretion.

Thus, although 24 h urinary cortisol has been used in several studies, several methodological questions remain that hamper the interpretation of the results. The aims of this study are twofold. First, we want to calculate the short- (two days) and long-term (two years) stability of 24-h UFC excretion. Second, we want to identify major sources of variability in 24-h UFC.

We use a large cohort study, in which urinary free cortisol was measured by liquid chromatography—tandem mass spectrometry (LC—MS/MS) analysis. Use of LC—MS/MS is recommended because this method offers advantages over immunoassays; the LC—MS/MS method is free of interferences from cortisol metabolites and conjugates and also eliminates drug interferences (Taylor et al., 2002). This is particularly important in this field of research, since reported cortisol levels vary widely between different laboratories, which is likely to be caused by the use of methods which may not distinguish between cortisol and its metabolites (Pearson Murphy, 1999; Fenske, 2004).

2. Methods

2.1. Population

Our investigation was performed in a cohort derived from the Prevention of REnal and Vascular ENd stage Disease (PREVEND) study, a major population-based cohort study investigating microalbuminuria as a risk factor for renal and cardiovascular disease. The recruitment of participants for PREVEND has been extensively described elsewhere (Pinto-Sietsma et al., 2000). All inhabitants of the city of Groningen, the Netherlands, between the ages of 28 and 75 years (85,421 subjects) were asked to send in a morning urine sample and to fill out a short questionnaire on demographics and cardiovascular history. A total of 40,856 subjects (47.8%) responded. After exclusion of subjects with insulin dependent diabetes mellitus and pregnant women, all subjects with an elevated urinary albumin concentration of $\geq$10 mg/l (N = 7768) together with a randomly selected control group with a urinary albumin concentration of $<$10 mg/l (N = 3395) were invited for further investigations (total N = 11,163). Finally, 8592 subjects completed the total screening program, rendering the PREVEND study cohort at T0 with a ratio of albuminuria-negative to albuminuria-positive subjects of 0.43. Because the PREVEND study population was enriched for albuminuria this oversampling for albuminuria was rectified in the current substudy. Albuminuria-negative participants were combined with a random sample of albuminuria-positive participants until a population-representative ratio of 3.08 was achieved. This resulted in a cohort of 2880 participants for whom at least one cortisol value was available at T1, for which data were collected between April 2001 and December 2003. These subjects were re-assessed (N = 2440, 84.7%) at T2, for which data were collected between December 2003 and October 2006. The median follow up period was 2.23 years (Inter Quartile Range (IQR) 2.11—2.43 years). All subjects gave written consent to participate in the study, which was approved by the local medical ethics committee.

2.2. HPA-axis function

Participants were asked to collect urine samples in a polypropylene container on two consecutive days at T1 and T2 (resulting in four urine collections for each participant). They were instructed to urinate into the container during the 24 h collection period, starting at 22:00 h, and store the sample in the fridge until delivery to the laboratory. Presumably incompliant subjects were excluded based on the following formula: (urinary creatinine [mmol/d] $\times$ 113)/(21 $\times$ body weight [kg]) of $<$0.7 (Knuiman et al., 1986), which proved to be the method of choice in a comparative analysis of exclusion strategies (Murakami et al., 2008). Numbers excluded were 733 (day 1) and 648 (day 2) for T1 (with an overlap of 439), and 690 (day 1) and 599 (day 2) for T2 (with an overlap of 428). Urinary free cortisol was measured by liquid chromatography—tandem mass spectrometry (LC—MS/MS) analysis (Taylor et al., 2002). The lower detection limit of the assay was 0.3 nmol/l. At low, middle, and high concentrations, intra-assay variation ranged from 1.3% to 2.4% while inter-assay variation ranged from 3.8% to 7.8%.

2.3. Medication use

Information on drug use was obtained from the IADB.nl, which contains dispensing information from 55 community pharmacies in the Netherlands, covering on average 500,000 persons annually (www.IADB.nl) (Visser et al., 2013). The database's pharmacy information includes, among others, name of the drug, anatomic—therapeutic—chemical (ATC) classification and date of prescription. With the exception of over-the-counter drugs and in-hospital prescriptions, all prescriptions are included regardless of prescriber, insurance, or reimbursement status. Medication records of patients are virtually complete because of high patient pharmacy commitment in the Netherlands (Monster et al., 2002). We extracted information on drug prescriptions from 100 days prior until 100 days after the date of the visit to our research facilities. We excluded participants using inhalation, local, gastrointestinal, or systemic glucocorticosteroids from the analyses (N = 550 for T1; N = 414 for T2; with an overlap of 218 participants using glucocorticoids at both T1 and T2).

2.4. Demographic and lifestyle factors

Sex, age, smoking, alcohol consumption, and exercise frequency were assessed by written self-report. Smoking was divided into current, past ($>$1 year), and never smoker. Exercise was indicated by the frequency of exercise (0 = not/hardly, 1 = once per week, 2 = twice or more per week). Alcohol consumption was divided based on the current mean number of alcoholic beverages into no alcohol use, 3 or less units per day, more than 3 units per day. One unit is equal to one serving of alcohol. Height and weight were measured and body mass index was calculated as the ratio between weight and the square of height (kg/m^2).

2.5. Psychological distress

Psychological distress was assessed using the Dutch version of the 12-item General Health Questionnaire (GHQ-12) (Koeter, 1992). Psychological distress scores were obtained by summing the Likert scores (0—1—2—3) on the 12 items. Missing values were imputed using Corrected Item Mean substitution if at least 50% of the items were completed (Huisman, 2000).

2.6. Renal function

Renal function (Chan et al., 2004) and urine volume (Mericq and Cutler, 1998) may be responsible for variance in 24-h UFC excretion. Glomerular filtration rate (GFR) was estimated using creatinine clearance ((1000/1440) * mean((urine volume day 1 * urinary creatinine concentration day 1), (urine volume day 2 * urinary creatinine concentration day 2))/serum creatinine concentration). Creatinine clearance is a good alternative for true GFR if information on 24 h urinary creatinine excretion is available (Traynor et al., 2006). The potential influence on serum creatinine levels of factors like meat consumption and exercise are taken into account by dividing serum creatinine by urinary creatinine excretion in the formula for calculating creatinine clearance (Traynor et al., 2006). As a result of tubular secretion of creatinine, creatinine clearance tends to slightly overestimate true

glomerular filtration rate. This is, however, a systematic error of fairly stable magnitude over the range of renal function, until advanced renal failure is reached (Traynor et al., 2006). Creatinine was assessed as described previously (Verhave et al., 2004). Blood sampling was performed in the fasting state, in the morning after the second 24 h urine collection.

2.7. Statistical analysis

All analyses were performed using IBM SPSS Statistics version 20. First, the 2-day average of total 24-h UFC was calculated based on compliant days (if only one compliant day was available, this value replaced the two-day average). To ensure normal distribution for parametric analyses, all cortisol values were log-transformed after which outliers which differed more than 3SD from the mean were removed ($N = 17$ (day 1), 17 (day 2), and 13 (2-day average) for T1; $N = 15$ (day1), 9 (day2), and 15 (2-day average) for T2). Second, we examined short and long term stability by calculating Pearson r for the 24-h UFC over two days, and for the 2-day average of 24-h UFC over two years. Finally, we investigated sources of variability by performing multivariable regressions. Multivariable linear regression analyses were performed separately for T1 and T2 to study independent effects of predictors on the 2-day average of 24-h UFC and reproducibility of results.

Table 1 Descriptives of the study population at T1 and T2. BMI: body mass index; GFR: glomular filtration rate according to creatinine clearance; 24-h UFC: 24-h urinary free cortisol; IQ R: inter quartile range; GHQ-12: 12-item General Health Questionnaire.

	T1	T2
Age mean (SD)	50.5 (10.9)	52.7 (10.6)
Sex (% female)	45.4%	41.7%
Smoking (%)		
Current	31.6%	30.8%
Past (>1 year)	40.9%	45.4%
Never	27.5%	23.7%
Alcohol (%)		
No alcohol use	19.2%	19.8%
3 or less units per day	76.6%	76.3%
More than 3 units per day	4.2%	4.0%
Exercise frequency (%)		
Not/hardly	53.2%	51.8%
Once/week	26.8%	25.0%
Twice or more/week	20.0%	23.2%
BMI (kg/m^2) mean (SD)	25.7 (3.6)	25.6 (3.5)
GFR (ml/min) mean (SD)	110.4 (22.4)	103.3 (21.0)
2-day average of urine volume (SD) (l)	1.79 (0.58)	1.84 (0.59)
24-h UFC median (IQ range) (nmol/24 h)	70.8 (49.6—99.7)	72.2 (49.8—99.8)
Psychological distress (GHQ-12) mean (SD)	11.1 (5.5)	10.9 (5.3)

3. Results

3.1. Study population

Table 1 shows the characteristics of our study population at the two visits (T1 and T2).

3.2. Short-term and long-term stability of cortisol values

24-h UFC values were relatively stable over two days and over two years. The correlation between 24-h UFC on day 1 and 2 was for T1 0.69 ($N = 1192$, $p < 0.001$) and for T2 0.72 ($N = 963$, $p < 0.001$). The correlation between T1 and T2 for the two-day average of 24-h UFC was 0.60 ($N = 972$, $p < 0.001$). To study the influence of follow up duration, we calculated T1—T2 correlations for the two-day average of 24-h UFC separately for participants with a follow up duration below and above the median. As expected, shorter follow up durations were associated with higher stability (Pearson $r = 0.63$, $N = 465$, $p < 0.001$ for follow up durations below the median, and Pearson $r = 0.57$, $N = 483$, $p < 0.001$ for follow up durations above the median).

3.3. Sources of variability: multivariable associations

Table 2 summarizes the multivariable models predicting the average 24-h UFC values at T1 and T2. Consistent relationships with 24-h UFC were found at both T1 and T2 for sex

Table 2 Multivariable models predicting 24-h urinary free cortisol. Standardized betas are provided. GFR: estimated glomerular filtration rate according to creatinine clearance; BMI: body mass index; GHQ-12: 12-item General Health Questionnaire.

	T1 ($N = 1556$)	T2 ($N = 1244$)
Female sex	−0.057*	−0.080*
Age	0.035	−0.040
Urine volume (l)	0.282**	0.276**
GFR (ml/min)	0.137**	0.179**
BMI (kg/m^2)	−0.065*	−0.047
Exercise frequency	0.014	0.050
Smoking	−0.054*	−0.044
Alcohol use	−0.020	−0.036
Psychological distress (GHQ-12)	0.009	0.022
Adjusted R square	0.100	0.115

* $p < 0.05$.
** $p < 0.001$.

(lower levels in females), urine volume and GFR (both positive). A lack of association was consistently found for age, exercise frequency, alcohol use, and psychological distress. Equivocal results were found for BMI and smoking, which both had a small but significant effect on 24-h UFC at T1 which could not be replicated at T2.

4. Discussion

This study shows that 24-h UFC is moderately stable in both the short (day to day) and the long term (two years). The most important sources of variability identified include urine volume and creatinine clearance, while also sex explained a small part of the cortisol variability.

To the best of our knowledge, we present the most extensive analyses of 24-h UFC in the literature. Strengths of this study are the large sample and the longitudinal dataset. The two measurement waves enable us to test reproducibility of the results in the same population using the same method. The repeated measurements also enable us to estimate long-term stability of 24-h UFC, while at the same time decreasing the risk of a chance finding while studying sources of variability. Another strength of our study is the use of LC—MS/MS enabling us to distinguish between urinary cortisol and cortisone, thereby avoiding the problem that urinary free cortisol values that have been determined by competitive binding assays may represent the sum of cortisol and cortisone (Fenske, 2004).

A limitation of our study is that additionally assessing the major urinary cortisol metabolites would have provided a more precise estimation of the cortisol secreted by the adrenal gland (Remer et al., 2008). Furthermore, 24-h UFC is sensitive to urine collection errors. We corrected for this by excluding presumably incompliant individuals (Knuiman et al., 1986), which proved to be the method of choice in a comparative analysis of exclusion strategies (Murakami et al., 2008). Excluding presumable non-compliant subjects is preferable over analyzing cortisol concentration in urine per unit of creatinine, since the latter does not take into account the point of the circadian rhythm (e.g. 08:00 h vs 20:00 h) at which the subject missed a urine collection, and the exact time point substantially influences UFC levels. Nevertheless, this led to the exclusion of a relatively large number of participants, which might have influenced the results if non-compliance was related to the amount of stressors encountered during the day. The final dataset might thus be composed of individuals collecting urine on days with low levels of stress, which would probably inflate the stability of the 24-h UFC, and underestimate its association with psychological distress.

Our results point to a reasonable day-to-day variation in 24-h UFC values. This has implications for the effect sizes that might be expected when associating cortisol to health problems. It also means that it is important to only use large cohorts for such studies in order to obtain sufficient power. Our data reveal some interesting sources of variability in cortisol values.

We found urine volume to be positively associated with total urinary free cortisol excretion. Previous studies finding a positive correlation between 24-h UFC and urine volume were not able to exclude the possibility that this increase in 24-h UFC with increasing urine volume was actually reflecting an increase in cortisone which was detected by the competitive binding assays used (Fenske, 2004). Our results prove the existence of a positive association between urine volume and 24-h UFC independent of increased cortisone formation. Thus, our study using LC—MS/MS provides an answer to this controversy in the literature.

Another important source of variability is GFR, which is positively associated with 24-h UFC and cortisol concentration. Our results are in line with a previous study showing that patients with moderate and especially severe renal impairment had significantly lower 24-h UFC rates than those with no or mild renal impairment (Chan et al., 2004). We show for the first time that similar associations can be found in the general population. Consistent unique effects on 24-h UFC were also found for sex, although the effect was small.

The question arises for which purposes urinary cortisol would be interesting. A disadvantage of using 24-h UFC as a measure of HPA-axis function is that it does not provide any information about the diurnal fluctuations in cortisol secretion, in contrast to serial salivary samples. Salivary cortisol could be used as an alternative, but is very sensitive to the exact timing of sampling. 24-h UFC has been criticized as a marker of cortisol secretion, even in mild forms of Cushings disease (Alexandraki and Grossman, 2011). Only about 2—3% of the cortisol produced per day can be detected as unconjugated cortisol in urine (Gatti et al., 2009). A by far greater percentage of the excreted cortisol is metabolized before it shows up in urine (beside the fraction that is excreted via colon and skin). Thus, even small changes in the complex metabolic process could easily halve, double or triple the assessed urinary cortisol level. This implies that even intraindividual stability over time of 24-h UFC may predominantly reflect the stability of the metabolic processes in liver and kidney and not the function of interest, i.e. adrenocortical activity. In addition, there are serious doubts about whether free cortisol is the only biologically active fraction; other candidates are cortisol which is only loosely bound to albumin, and the corticosterone—CBG complex (Levine et al., 2007). However, studies do suggest that urinary cortisol reflects blood levels, as shown by a strong association between plasma and excreted cortisol in an early study (Beisel et al., 1964) and high intraclass correlation between 24-h UFC and salivary and serum cortisol in a more recent study (Neary et al., 2002). The use of urinary cortisol for psychobiological approaches is supported by a single-subject study in which 24-h UFC was assessed for 63 days, which revealed that stressful incidents were reflected in subsequent changes in urinary cortisol (Schubert et al., 2012). Another study showed that urinary cortisol increases under influence of stress associated with a parachute jump (Plenis et al., 2011). As mentioned, several interesting associations between psychiatric and somatic health problems and urinary cortisol have been published previously (Oldehinkel et al., 2001; Vogelzangs et al., 2010; Tak et al., 2011). We did not show any associations with psychological distress, as assessed with the GHQ-12. This questionnaire assesses psychological distress in the previous weeks. It remains possible that the day of sampling was not representative for the levels of psychological distress in the last weeks, which might explain the lack of association between 24-h UFC and psychological distress.

We conclude that 24-h UFC is a suitable measure to study HPA-axis activity in epidemiological cohorts. When interpreting the data, the effects of urine volume and glomerular filtration rate on 24-h UFC could be taken into account. Studies that collect time series of 24 h UFC within an individual over several weeks would be interesting to further unravel cortisol stability, and to study whether this variability differs between individuals. It remains important to use assays that are not influenced by cross-reactivity, and to ensure a rather strict and exact urine collection. The usefulness in the study of psychobiological research, and the exact meaning of the measure, deserve further study.

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Conflict of interest

None declared.

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